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## Identification of Rare Florilegia in Lloyd Library

Corinne Miller Simons

(*Lloyd Library and Museum, Cincinnati, Ohio*)

*"Books are the legacies that a great genius leaves to mankind . . ."* Addison

For many years there has been in Lloyd Library among a collection of rare books an unusual item consisting of 295 hand-colored flower plates bound in plain red buckram. No text, title-page, imprint, colophon, index or table of contents accompanies this volume. These plates are numbered but not arranged in any apparent logical manner. Drug, ornamental and economic plants are depicted. The plates measure 8¾ x 14½ inches in size. The paper they are printed on is of a fine texture, slightly ridged and has the appearance of being hand-made. Everyone who saw this collection said that it undoubtedly is old and rare, but no one was able to identify either the unknown artist or the equally unknown author. The plates contain names in pre-Linnean Latin in medium uneven sloping script and German names in German script. Common names in English were inserted in pencil. The numbers and initials which appeared in the upper right hand corner are not alphabetical or numerically arranged.

These plates were given to Lloyd Library about 1931 by Mrs. Anne Burt Norton, a great-great-niece of Daniel Gano. They were formerly the property of Dr. O. D. Norton, of Cincinnati, Ohio who had received them in 1873 from Major Daniel Gano, an early settler of this area, who autographed the fly-leaf. An annotation was dated June 1820 and it was believed that the gardener of Daniel Gano brought these from Germany. This probably was the first book of botanical illustrations brought to Cincinnati or the Northwest Territory. In February and March of 1958 the author did some extensive research on flower plates and identified these volumes as part of a work by Georg Wolfgang Knorr of Nuremberg, Germany in 1770–72. The original set of 403 plates was published by Knorr in his *THESAURUS REI HERBARIAE HORTENSISQUE UNIVERSALIS EXHIBENS FLORUM, HERBARUM, ARBORUM, FRUTICUM, ALIARUMQUE PLANTARUM PRORSUS NOVAS ET AD IPSOS DELINEATAS DEPICTASQUE ARCHETYPOS NATIVIS COLORISUS . . .*

*ALLGEMEINES BLUMEN-, KRAUTER-, FRUCHT-, UND GARTENBUCH . . . USUM IN OFFICINIS PHARMACEUTICIS, VITA COMMUNI ET MEDICINA, PROVT SINGULA HAEC NOVA DOCUIT APPLICATA OBSERVATIO ET ARCHET VPORUM EXACTA CONTEMPLATIO.* The first volume, under this title, was written by P. F. Gmelin, Bose, Wittenberg and G. R. Böhmer, Wittenberg. Knorr was born in Nuremberg, Germany on December 30, 1705 and died there on September 17, 1761. He was an artist, draughtsman and engraver. He was a turner until the age of eighteen following his father's trade. He made numerous portraits and engravings separately and for illustrating books, including those for Scheuchzer's *Physica Sacra* which gave him a great interest in science. He also engraved some of the portraits for Friedr. Roth-ScHoltz, *Icones Bibliopolarum et Typographorum* 1726–1742. His *Views of Nürnberg and its Neighborhood* was published in 1737. Nissen says Knorr's principal work was the *Thesaurus*. His etchings and engravings were colored by hand. Knorr's heirs published several editions after his death. He is well known for his *Auserlosenes Blumen-Zeichinbuch für Damen* and numerous illustrations of plants and animals after Dürer, Kilian, and others. Nuremberg, after London, was the most important center of botanical art of the day, and the patronage of Trew who published one of the most decorative florilegia of the mid-eighteenth century, brought into being a number of local artists. These flower books are of an age which can never return. The florilegia or flower books were compiled for the horticulturist rather than the botanist, though it is difficult to draw a precise line of demarcation between scientific and non-scientific purposes. This type of book was developed towards the close of the sixteenth century when flowers first began to be extensively cultivated for their beauty rather than their utility and reached its highest popularity in the seventeenth century.

Philipp Friedrich Gmelin was a botanist of Tübinger, Germany, and member of a renowned family of physicians and botanists. In 1827 he published his dissertation *Uber der Winden der Pflanzen.* Georg Ralph Böhmer (1723–1803) was an eminent professor of botany and anatomy in the University of Wittenberg. He was the author of a great many treatises on every branch of botanical science, and much admired for his original thoughts, perspicuity of method and extensive knowledge. Volume two contains the plates which were painted by Knorr. These have a separate title-page: *REGNUM FLORAE, DAS REICH DER BLUMEN MIT ALLEN SEINEN SCHONHEITEN NACH DER NATUR AND IHREN FARBEN VORGESTELLET MIT TITELKUPF U. 301 KOL.* (*A–Z MIT 199 UND A–V MIT 102 KUPF.*). An index and table of contents was also published in the set. Dunthorne's *Flower and Fruit Prints of the 18th and early 19th century* gives a careful description of the plates, and states, "The following are the important plates: Stapelia (in pot), Pears, Apricot, Cereus, Crown Imperial, Ketmia (2 plates), Iris (3 plates), Lily, Narcissus, Helleborne, Quince, Rose (3 plates), Single Peony, Poppy, Cyclamen in pot, Parrot Tulip (4 plates), Wall flower, Heavenly Blue Convolvulus (and 5 other plates), Amaranthus, Auricula (in pot),

Pepper, Hops." Some of the plants are illustrated in decorated flower pots as the Stapelia on plate "F 4:" Cyclamen on plate "S 16" and Auricula on plate "A 10" in "P II." The plates are divided in two parts. Part one is arranged alphabetically and is indicated by a letter and plate number only. Part two starts the alphabet over again and is indicated by the designation "P II" and a letter and plate number. The plates are not consecutively numbered nor placed in sequence. In the first part the letters refer to the initial letters of the German common name, e.g. *Eiche*—"E 2"; in "P II" to initial letter of the Latin scientific name "O 1"—*Opuntia*. The colors appear to be as vivid and bright as the day they were made. Flowers, leaves and stems are shown in the illustrations. On some plates fruits are shown, as well as parts of the inflorescence. Other data in Blunt's *The Art of Botanical Book Illustration* confirmed this identification. Likewise Nissen's *Die Botanische Buchillustration* give good descriptions of the plates and text. Blunt states that "Knorr's Thesaurus (1780) shows that the influence of Trew was still alive in South Germany." Dunthorne indicated that only one copy was known to be in existence in this country and that was in the possession of the United States Department of Agriculture Library, 1780 edition. Upon examination of a microfilm obtained from this library the date clearly shows it to be 1770. It is hoped that with the publication of this article possibly other copies of Knorr or separate prints in the possession of private collectors may come to the attention of botanists and be listed in bibliographies.

Still the conclusions reached were not entirely proved, though certainly everything pointed to the above identification. It was then discovered that the watermark in the plates was clearly visible when held up to a light, as *J. Hoonig & Zoonen*. Some plates showed a fleur-de-lis and others the name *I. Villedary*. In Hunter's *Papermaking, the History and Technique of the Ancient Craft* is the statement that Hoonig & Zoonen were well-known Dutch papermakers of Zaandyk, Holland, 1737–1787. It was they who made the paper for the Declaration of Independence, 1776. They used the fleur-de-lis as a watermark having been adopted by them from France where the insignia referred to the direct descent of the Dutch Royal House of Burgundy from the Kings of France. Examination of Churchill's *Watermarks* showed the fleur-de-lis was known in the paper industry as the *Strasburg Lily* and that I. Villedary was a famous French papermaker who made paper for Hoonig and Zoonen of Holland. The name or initials of Villedary as a watermark covered a period of almost 150 years, 1668–1812. The first French papermarkers of this name made paper for four Dutch manufacturers or "factors." The name appears on innumerable manuscripts and official papers in the public archives and libraries of England and Holland. It is not known whether Villedary worked in conjunction with other papermakers, or that they made use of his name or initials only, which had become a hallmark of excellence in the trade. The paper industry in Europe and Holland is one of the results of wars, and as such with all their ramifications offer a fascinating story not only in history but in the arts and sciences.


FIG. 1. Title page from photostat of copy in U. S. Department of Agriculture Library.


FIG. 2. *Africanische Frittillaria.*


FIG. 3. Plate S 16. *Cyclamen.*

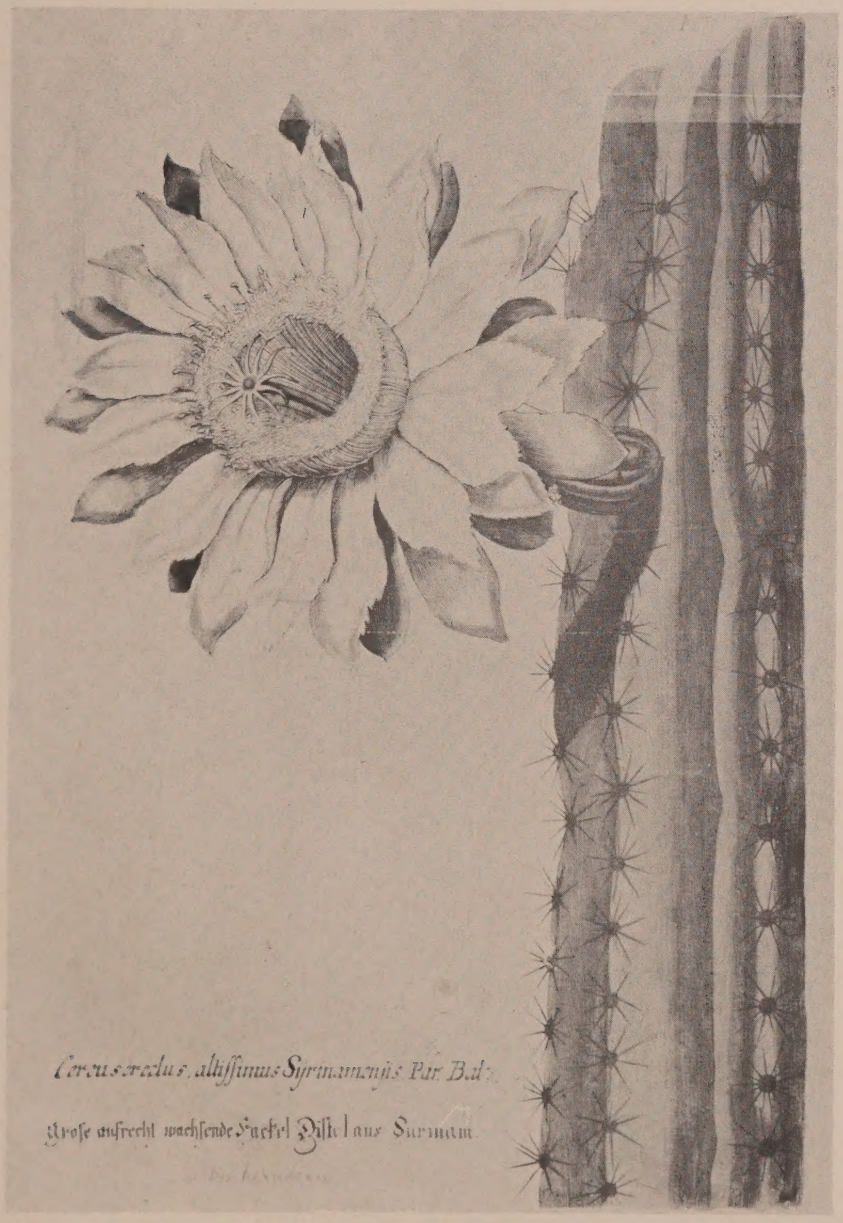


FIG. 4. Plate F 7. *Cereus erectus.*


FIG. 5. Plate A 10. *Aloe Africana*.

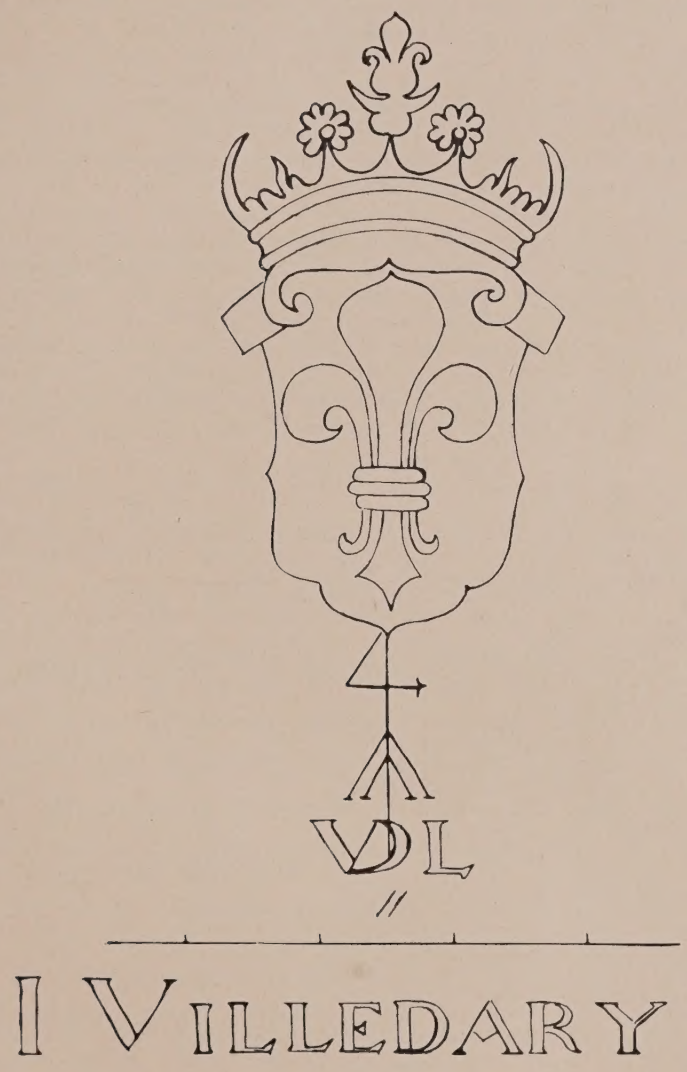


FIG. 6. Drawing of *Strasburg Lily* in Heraldic Shield in watermark of paper made by I. Villedary.

Photostats were obtained from the United States Department of Agriculture Library copy and in every instance plates number "F 7" *Cereus erectus;* "F 4" *Stapelia denticulis ramorum;* "S 16" *Cyclamen foliis cordatis;* "A 10" *Auricula ursis flore* matched the Lloyd Library copy exactly. Microfilms were also made for the missing plates and for the entire volume of text including indexes and table of contents. Therefore, it is reasonable to assume that the Lloyd copy is the second known copy in the United States. Certain foreign catalogues locate copies in European Libraries, but some recent ones do not include them. Knorr was known to have also painted plates for other numerous volumes, a list of which appears in Thieme-Becker's *Künstler-Lexicon.* Firstly, it is gratifying to state that the florilegia which had so long remained unidentified is now properly catalogued and placed with the volumes of similar classification. Secondly, these plates by Knorr are not only rare but priceless. Thirdly, they are in the possession of Lloyd Library where they are preserved for posterity. Truly it can be said with Keats that "A thing of beauty is a joy forever," and with Cavally that "A Library guards the light passing genius left burning."

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# Preliminary Growth and Nutrition Studies of Amanita Muscaria in Submerged Culture

Leon R. Pacifici, Ralph N. Blomster, Henry J. Lukowski and Arthur E. Schwarting

(*Pharmacognosy Research Laboratories, Pharmacy Research Institute, University of Connecticut, Storrs*)

The genus *Amanita* (Fam. *Agaricaceae*) is represented by a number of poisonous species. Perhaps the best known of these is the commonly occuring mushroom *Amanita muscaria* L., noted for its toxic quaternary amine, muscarine. The differing toxic properties of other species have been traced to a variety of chemical components.

Muscarine, although isolated in the last century (1) was only recently proved by Kögl and co-workers (2) to be a derivative of tetrahydrofuran. Although muscarine has no therapeutic use, its classic parasympathomimetic action preserves it as a pharmacological tool (3). The historical aspects of muscarine, including its pharmacology, have been reviewed by Bowden and Mogey (1). Balenović and Štefanac (4) have developed a simplified ion-exchange procedure for the separation of the quaternary bases of *A. muscaria*, particularly choline and muscarine. One of the previously unidentified bases has been shown by Kögl (5) to be muscaridine, a hexane derivative.

The highly toxic properties of *A. phalloides* have been traced to the cyclic peptides phalloidin, phalloin and $\alpha$, $\beta$ and $\gamma$ amanitin. The chemistry and toxicology of these toxins have recently been reviewed by Wieland and Wieland (6). Brady and Tyler (7) have presented chromatographic evidence for as yet unidentified alkaloids in *A. pantherina*. Bufotenine, an hallucinogenic indole derivative, has been isolated from *A. mappa* by Wieland and Motzel (8). Wilkins (9) has investigated a large number of basidiomycetes for antibiotic activity and has found such activity in some *Amanita* species.

The growth and nutrition of basidiomycetes in submerged culture received early attention (10) during efforts to produce mycelium of *Agaricus campestris* having the typical "mushroom" flavor. A recent review (11) on this subject notes similar studies with *Morchella*, *Lepiota* and *Coprinus* species. Although Block and co-workers (12) have studied vitamin content, strong interest in the production of drug substances by mushroom mycelium in submerged culture has been lacking.

Because of the variety of physiologically active components already discovered in the fruiting bodies of *Amanita* species, it was considered desirable to study the capacity of submerged cultures of these organisms to produce substances having possible therapeutic value. The present study deals with a preliminary investigation of the factors affecting the growth and nutrition of *A. muscaria*. Separate investigations for antibiotic production and proto-alkaloid and alkaloid accumulation are in progress.

## MATERIALS AND METHODS

Cultural Methods.—A strain of *Amanita muscaria* designated AM–4, selected for its ability to grow readily in submerged culture, was maintained on slants of Sabouraud dextrose agar at 3–5°C.

The basal medium used in this study was composed of the following; g per 1: dextrose, 20; Neopeptone, 20; $NH_4NO_3$, 5; $KH_2PO_4$, 2; and $MgSO_4.7H_2O$, 1. Three modifications of this basal medium were effected in the course of the nutritional studies. The first lowered dextrose concentration to 1%, the second omitted Neopeptone, and the third omitted Neopeptone and substituted either 6.7 g $NH_4Cl$ or 12.7 g $KNO_3$ for the $NH_4NO_3$.

Growth of the organism was carried out in 500 ml Erlenmeyer flasks, each containing 100 ml of medium. The flasks were shaken at 250 rpm on a rotary shaker[1] that described a circle 2 inches in diameter, or at 114 excursions per minute on a reciprocating shaker having a 2-inch stroke. Both shakers were operated in controlled temperature rooms.

Inoculum was obtained by aseptically transferring a small piece of mycelium from a slant into a flask containing basal medium and allowing the flask to shake until moderate growth ensued. Mycelium was then transferred from this stock flask into a series of experimental flasks using a 0.8 ml perforated transfer cup. The flasks were previously plugged with non-absorbent cotton and autoclaved at 15 lb. pressure for 15 minutes.

Analytical Methods.—Growth weight estimations were made by collecting and washing the mycelium in tared glass fritted disc crucibles followed by drying at 80°C for 48 hours. When thickening of the medium inhibited filtration, the mycelium was collected and washed by centrifugation, followed by quantitative transfer to the crucibles and drying as described above. Samples of the undiluted filtrate or centrifugate were frozen and stored at −10°C prior to analysis. The volume of the culture medium was measured at the time of samplying to determine the loss resulting from evaporation. All of the analytical results were corrected to a volume of 100 ml.

Total carbohydrate was determined colorimetrically[2] with anthrone according to the method described by Morris (13).

Total nitrogen was determined by a modified Kjeldahl-Nessler method. Digestion of 2 ml aliquots of diluted culture filtrate or centrifugate was accomplished in 35 ml calibrated digestion tubes supported on a rotary digestion rack.[3] After addition of one drop of 1 N $H_2SO_4$ together with a carborundum chip, the sample was reduced to a small (ca. 0.1 ml) volume by boiling. Two ml of conc. $H_2SO_4$ containing 2 mg of salicylic acid were added and the mixture heated to incipient boiling followed by cooling. This pretreatment was necessary to accomplish the ultimate conversion of nitrate to a determinable form of nitrogen. The findings of Dickinson (14) have indicated that omission of the customary thiosulfate reduction results in a small

[1]Model G53, New Brunswick Scientific Co., New Brunswick, N. J.
[2]Bausch and Lomb Spectronic 20 Colorimeter.
[3]American Instrument Co., Inc., Silver Spring, Md.

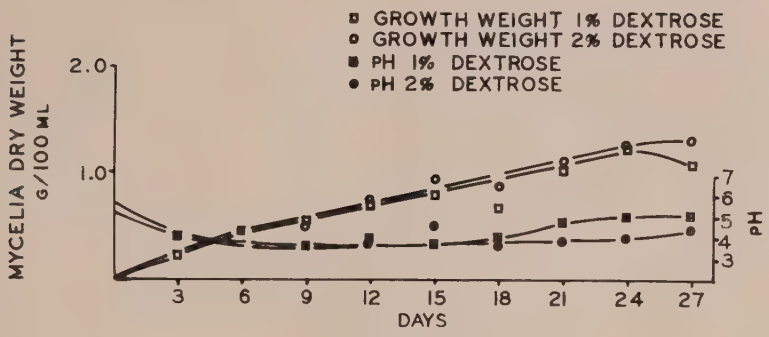


FIGURE 1
GROWTH & pH AT 22° C.

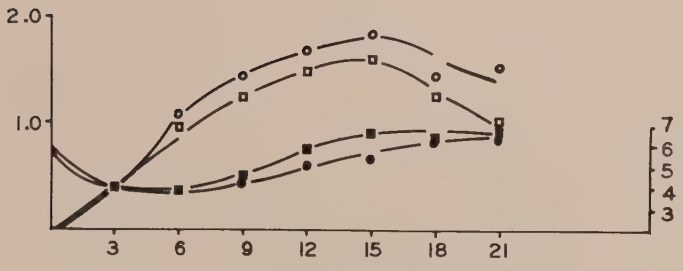


FIGURE 2
GROWTH & pH AT 25° C.

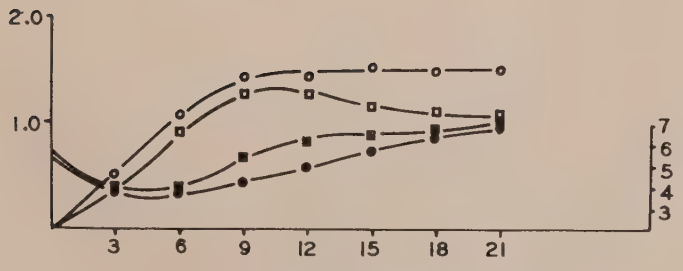


FIGURE 3
GROWTH & pH AT 28° C.

error, not of practical significance in the present work. After cooling, 1 ml of mercury catalyst (15), 1.3 g of $K_2SO_4$ and a carborundum chip were added. The catalyst was prepared by dissolving 5 g of HgO and 10 ml of conc. $H_2SO_4$ in sufficient distilled water to make 100 ml. The sample was digested for 1 hour after clearing (16). Determination of residual $H_2SO_4$ after a typical digestion gave a ratio of ml $H_2SO_4$:g $K_2SO_4$ of about 1.25. This ratio has been termed the "acid index" (17) and is the controlling factor of digestion temperature. The ratio used maintains the elevated temperature recommended by Lake (18) to assure quantitative recovery of resistant heterocyclic nitrogen. After digestion, zinc dust was added (15) as 1 ml of a 20% v/v suspension in distilled water. The colorimetric determination was made according to Polley (15) with adjustments in quantities as required by apparatus and nitrogen concentration. Results were calculated as mycelial nitrogen according to Jarvis and Johnson (19).

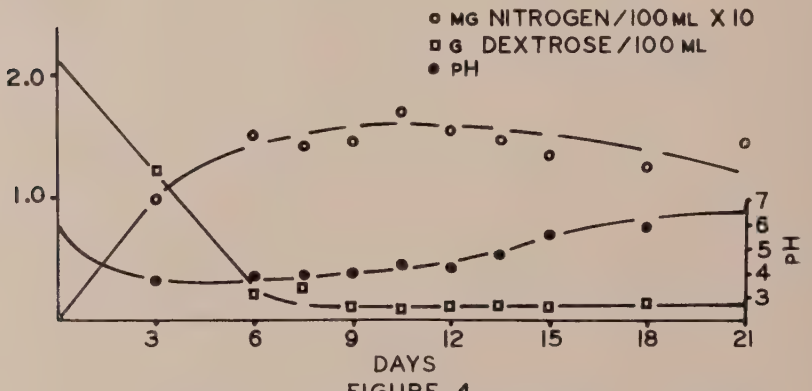


FIGURE 4
GROWTH & NUTRITION IN THE BASAL MEDIUM AT 28°C.

## EXPERIMENTAL AND RESULTS

Temperature.—The effect of temperature on growth was determined by culturing the organism in the basal medium and in the 1% dextrose modification at 22°, 25°, 28°, and 31°C on the reciprocating shaker. Flasks of each medium were harvested in quadruplicate at 3 day intervals and the pH and growth weight in each were determined. The experiments were continued until the dry weight of mycelium remained constant or started to decline. Growth was so slow at 22°C that the experiment was terminated after 27 days even though maximum growth had not been reached. The mean values of pH and growth weight in media at 22°, 25° and 28°C are presented in figures 1–3. The results of the study conducted at 31°C indicated that little or no growth had taken place.

Nutrition.—Carbohydrate utilization and incorporation of nitrogen into mycelium grown in the basal medium at 28°C on the reciprocating

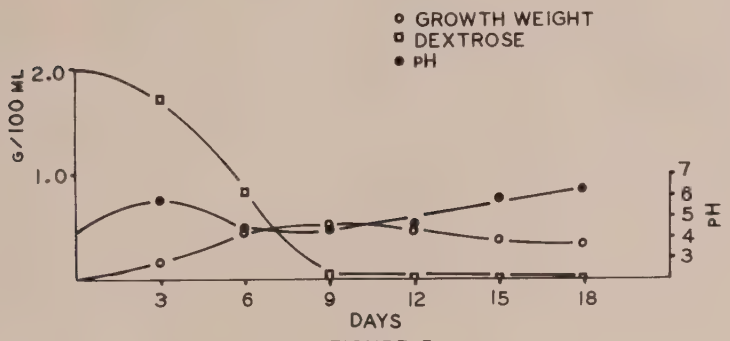


FIGURE 5

GROWTH & NUTRITION IN INORGANIC-NITROGEN MEDIA–AMMONIUM NITRATE

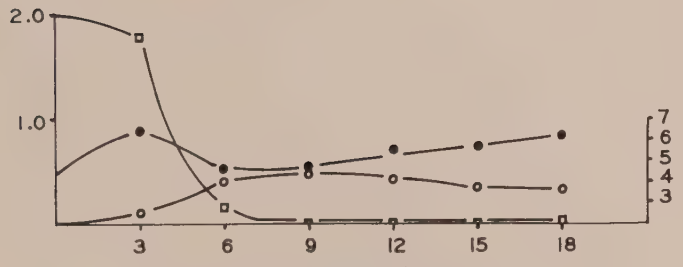


FIGURE 6

GROWTH & NUTRITION IN INORGANIC-NITROGEN MEDIA–POTASSIUM NITRATE

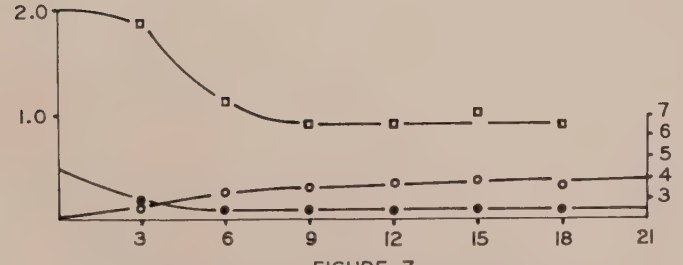


FIGURE 7

GROWTH & NUTRITION IN INORGANIC-NITROGEN MEDIA–AMMONIUM CHLORIDE

shaker were studied by determining total carbohydrate and soluble nitrogen in the filtered or centrifuged both at various times during growth. Flasks were harvested in triplicate at 1½ or 3 day intervals and their contents were pooled prior to analysis. Figure 4 shows the results of these analyses together with the pH values of the pooled filtrates.

The effectiveness of the various inorganic forms of nitrogen in supporting growth of the organism was also studied. In an effort to avoid the introduction of organic nitrogen or essential growth factors into the inorganic nitrogen media, the inoculum used in these experiments was prepared by two successive cultures of the mycelium in basal medium containing no Neopeptone. Growth of the organism in the inorganic nitrogen media was conducted at 22°C on the rotary shaker. Flasks of each medium were harvested in quadruplicate at 3 day intervals and the filtrates were pooled for analysis. Mycelial dry weight, total carbohydrate and pH were determined and are presented in figures 5–7.

A characteristic deep red pigmentation appeared during growth of the organism in the basal medium, but did not appear when the Neopeptone was excluded from the medium. Reduction of the Neopeptone concentration to 1% resulted in a distinct decrease in the concentration of this pigment. The pigment failed to appear regularly in the presence of Neopeptone when cultures were incubated on the reciprocating shaker at 31°C. Some flasks showing no pigmentation after one week were removed and placed on the rotary shaker at 25°C. Rapid growth ensued, but no pigment was formed.

With the media containing $NH_4NO_3$ or $KNO_3$ as the sole source of nitrogen, a yellow to greenish-yellow pigmentation was observed. However, little or no pigmentation occured in the medium containing $NH_4Cl$.

## DISCUSSION

Temperature.—The mycelial growth rates at 25° and at 28°C were nearly identical for the first 9 days of the experiment. After this period, however, the rate of growth at 28°C showed a rapid decrease whereas that at 25°C decreased slowly over a period of several days. With the basal medium containing 2% dextrose, the yield of mycelium at 28°C reached a maximum of 1.56 g per 100 ml after about 12 days. A higher maximum, 1.86 g per 100 ml, was obtained at 25°C after 15 days. The weights of mycelium obtained with the basal medium containing only 1% dextrose were somewhat lower at all temperatures. However, no conclusions can be drawn from this observation since the Neopeptone contributed considerable amounts of both carbon and nitrogen whose availability to the organism is not known.

The pattern of pH change was essentially the same at 22°, 25° and 28°C; an initial decrease during the period of most rapid growth was followed by a slow increase as growth subsided.

A substantial reduction in the rate of growth and pH change was observed in the experiment conducted at 22°C. The results obtained at 31°C indicated that this temperature permitted only meager growth of the organism.

Nutrition.—Dextrose appeared to be utilized equally well in the basal medium and in the media containing inorganic nitrogen as the sole source of nitrogen.

The initial rate of growth in the inorganic nitrogen media was very similar to that observed with the basal medium at the same temperature. However, the total growth was only about 30% of that obtained with the basal medium at 28°C. This is probably due to the greater amount of carbon and nitrogen supplied by the Neopeptone.

A comparison of the relative effectiveness of inorganic and organic nitrogen in supporting growth of the organism is not possible because the media did not contain equivalent amounts of either carbon or nitrogen. However, the initial rise in pH with the medium containing $NH_4NO_3$ as the sole source of nitrogen indicates that the organism shows some preference for the utilization of nitrate-nitrogen over ammonia-nitrogen.

The decrease in pH to a value of 2.5 in the medium containing $NH_4Cl$ resulted in cessation of growth and dextrose utilization by the organism.

## SUMMARY

A preliminary study of the growth and nutrition of *Amanita muscaria* in submerged culture was performed. The effect of temperature as well as various nitrogen sources on the behavior of cultures of a strain of this organism, designated AM–4, was determined.

Maximum growth was obtained at 25°C in a medium containing Neopeptone. Growth was also obtained in media containing ammonia or nitrate-nitrogen as the sole source of nitrogen.

## ACKNOWLEDGMENT

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# The Non-isotopic Precursor Feeding Technique in the Study of Alkaloid Biosynthesis

V. E. Tyler, Jr.

*(Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle 5)*

Although grafting experiments aimed at elucidating the site of alkaloid synthesis in plants were carried out prior to the twentieth century (1), the results of the first systematic feeding experiments designed to establish the identity of alkaloidal precursors were published in 1932. In a series of three papers entitled "Zur Bildung der Betaine und der Alkaloide in der Pflanze," G. Klein and H. Linser (2–4) laid the foundation for all subsequent studies of alkaloid biosynthesis and applied a methodology to this type of investigation which is still employed today.

The injection and infiltration of various compounds into living plants have been practiced since very early times. The history of the practice has been outlined and its application as a physiological method discussed in a review by W. A. Roach (5). Nevertheless, Klein and Linser were the first to apply such a procedure to the study of the biosynthesis of betaines and alkaloids. In the first paper of their series, these authors point out that the feeding method would appear to be a very useful one since the administration of large quantities of precursor would lead one to expect an increase in end product, provided, of course, that the hypothetical reaction sequence is correct. It was considered desirable to inject or otherwise infiltrate the hypothetical precursors directly into the plant instead of incorporating them into a nutrient solution intended to be taken up by the roots. Such direct incorporation would obviate the necessity of conducting the experiment under sterile conditions to prevent the undesirable actions of microorganisms. Root feeding was also considered to be undesirable, according to the authors, because amino acids are taken up very slowly by the root and are either assimilated by that organ or subjected to oxidative deamination there.

In order to avoid these supposed difficulties, Klein and Linser employed a method in which the precursor was incorporated directly into the aerial portion of the plant by immersing the cut stem into the nutrient solution. Uptake could be facilitated by the use of reduced pressure. A more elegant procedure which yielded superior results was the injection of a 0.1–0.2% solution of the neutralized amino acid or its derivative into the hollow stem of an intact plant. Two small holes were made in the stem of the plant, one just above a leaf node, the other just below the next higher node. The liquid was injected into the lower hole until it began to flow from the upper, and then both holes were closed with grafting wax. In addition to the postulated precursors, other compounds, principally amino acids but also inorganic salts, were injected into plants which served as controls. These were employed to show that only specific precursors caused any appreciable increase in the desired end products. Two or three injections were

generally made at intervals of eight to fourteen days, and the plants were harvested and subjected to analysis approximately one month after the treatment commenced.

On the basis of experiments of this type carried out in dahlia, it was concluded that feedings of proline, glutamic acid, pyrrolidone-carboxylic acid, ornithine, δ-aminovaleric acid, α-amino-δ-hydroxy-valeric acid, and α-hydroxy-δ-aminovaleric acid all resulted in an increased formation of trigonelline. Methenamine in the presence of proline resulted in considerably greater increases than were observed with the amino acid alone. Unfortunately, niacin, which modern research has shown to function as a precursor of trigonelline (6), was not tested. From the results of similar experiments conducted with *Stachys palustris*, *S. recta*, and *Galeopsis ochroleuca*, the authors concluded that proline serves as a precursor to stachydrine. Recent experiments employing isotopically labeled compounds have not been able to obtain evidence for the conversion of either ornithine or proline into stachydrine, but N-methyl proline does serve as a precursor of stachydrine in alfalfa seedlings (7).

Klein and Linser next immersed the ends of cut shoots of *Nicotiana rustica* and *N. havanensis* in a 10% proline solution and determined the nicotine content after nine to fourteen days. Increases in alkaloid content as high as 190%, calculated on a dry weight basis, were taken as evidence that proline also serves as a precursor to nicotine.

James (8) has analyzed these feeding experiments and concluded that this theoretically simple technique is fraught with pitfalls, based principally on sampling difficulties. Since conventional methods of analysis require the destruction of the sample analyzed, variations in alkaloid content caused by precursor feeding can be detected only by comparison with suitable controls. Different plants or even different groups of plants are seldom identical in all respects, and the selection of a proper basis of comparison is difficult. Both Dawson (9) and James (10) are in agreement that the most meaningful expression of analytical data is in terms of absolute quantities per organ or per plant instead of percentage of fresh or dry weight.

Klein and Linser recognized the variations in fresh weight over extended periods of time, which rendered this an unsuitable basis for comparison. They failed to recognize the fact that injections of amino acids are known to stimulate respiration, causing an increased consumption of carbohydrate which would result in a decrease in dry weight of a particular volume of tissue. The supposed increases in trigonelline in their experimental plants could have arisen by such variations in dry weight. Furthermore, in most of their trials the quantity of base present at the end of the experimental period was lower for both the test plants and the controls than at the beginning. Therefore, what was termed alkaloidal synthesis was in actuality only a lesser decrease in content in the experimental plants as compared with the controls.

The results of their experiments concerned with nicotine biosynthesis are more difficult to explain as variations in the dry weight of samples since differences as high as 190% were reported. This is all the more amazing in view of the recent evidence that the root of the tobacco plant is the *Hauptbildungstätte* of nicotine. In fact, the consistent

ability of a number of investigators to obtain apparent increases in alkaloid content in detached plant organs which are incapable of synthesizing any appreciable quantity of alkaloids was one of the factors which led the present writer to question the suitability of the method. Obviously, the utility of the feeding or injection technique cannot be judged from these initial experiments of Klein and Linser. Further examination reveals even more questionable features of the original hypothesis upon which the method is based.

Addition of a precursor would result in an increase in product only if the quantity of the precursor is the limiting factor in the biosynthetic pathway. The presence or state of activity of enzyme systems required in the conversion might just as conceivably be limiting, in which case addition of the proper precursor would not have the postulated effect. The degree to which alkaloid accumulation is controlled by genetic factors remains largely unknown, although it has been shown that nicotine synthesis in the tobacco plant is very stable and not easily influenced (11).

The question then arises, if the results of more recent feeding experiments, when compared to those accepted pathways of biosynthesis as determined by radioisotope studies, have shed any further light upon the utility of this classical method which would either justify its employment in modern experimentation or require it to be deleted from the methodology of alkaloid investigation. A critical examination of such experiments should reveal such factors as the best method of sampling and comparison of data, suitable techniques for precursor administration, and finally, it should settle the question as to whether or not the availability of certain precursors is actually the limiting factor of alkaloid biosynthesis in plants. The experiments must be analyzed carefully to determine if the plants or parts of plants employed were actually capable of alkaloid synthesis at the stage of development in which they were employed and under the conditions tested.

Numerous isotopic investigations have yielded considerable information as to the biosynthetic precursors of the tropane alkaloids. These studies have established with certainty (12) that ornithine is the precursor of the tropine nucleus of hyoscyamine, but the ability of putrescine to function in this capacity remains unproven. Methionine has been shown to serve as a methyl donor for the N-methylation of hyocyamine, and phenylalanine appears to function as a direct precursor of tropic acid (13). The tropane alkaloids are principally products of root metabolism, although small quantities are formed in young shoots and in the developing fruits.

Several interesting non-isotopic precursor feeding experiments have also been carried out with this group of alkaloids. Cromwell (14) injected solutions of potassium nitrate, ammonium sulfate, asparagine, urea, nitrogenous bases, and amino acids into plants of *Atropa* (two years old) and *Datura*, with the object of observing the effect on the plants and on the production of alkaloids. After petiolar and stem injections proved unsatisfactory, the author devised an ingenious method which unfortunately is not described in sufficient detail in his publication. It was determined that injection through the stump of a stem was the most satisfactory procedure for introducing solutions

into *Atropa* plants, while branch injection was used in the case of *Datura*.

The solution was contained in a liter aspirator bottle suspended three feet above the ground and was conveyed to the cut end of the stump through rubber tubing. With good transpiration, as much as 400 ml of solution passed into an *Atropa* plant in three days; 100 ml was the average volume passed into each *Datura* plant. Samples of leaves and roots were taken before and after the injection period. Preliminary experiments with eosin solution indicated that such injections reached the greater part of the root and shoot systems of the plants. Two percent solutions of glucose and 0.25% of all other substances were used, and the duration of injection was seven days. None of the substances injected at these concentrations appeared to injure the plants.

Injections of arginine with glucose, putrescine alone and with glucose, hexamine with glucose, and formamol with glucose gave substantial increases (>0.05%, dry weight basis) in the hyoscyamine content of *Atropa belladonna*. Putrescine with glucose and formamol alone gave the best results with *Datura stramonium*. In neither plant did arginine alone cause any appreciable increase in alkaloid concentration, either in the leaves or in the roots.

One valid criticism which may be leveled against this experiment is that of improper sampling. Results are reported as a percentage of dry weight, not as quantities of alkaloid per plant or per organ. Furthermore, the experiment fails to differentiate between direct and indirect precursors, glucose and formamol giving better results in *Datura* than arginine plus glucose or putrescine plus glucose. Even the largest increases in alkaloid content are comparatively slight (putrescine plus glucose in belladonna leaf, control = 0.44%, experimental = 0.70%, difference = 0.26%), but this is perhaps to be expected from the known limited ability of the plant parts employed to carry out alkaloid synthesis.

The significance of the experiment remains even more in doubt because the only isotope feeding experiment with putrescine has yielded negative results; however, Mothes is of the opinion that this may have been due to the fact that the plant was not synthesizing any alkaloid during the course of the experiment.

James (10) also investigated the influence of amino acid precursors on the biosynthesis of the belladonna alkaloids. Petioles of excised leaves were dipped into feeding solutions which were replaced by fresh solutions every twenty-four to forty-eight hours. Total duration of the experiments was three or four days because control leaves in water or sucrose solution were found to remain healthy for this period of time. Leaves were dried and assayed, and since samples were arranged to have both equal numbers of leaves and equal initial fresh weights, it was immaterial whether the amounts of alkaloids were calculated per leaf or by initial weight.

Solutions of 0.01 *M* L-arginine and 0.01 *M* L-ornithine supplied in addition to 1% sucrose caused increases in the mg of alkaloid per leaf both over the initial value at the time of picking and over the value for leaves supplied with 1% sucrose only. The increases were small but

statistically significant, and neither 0.01 *M* proline nor 0.01 *M* ammonium sulfate caused similar increases. It was concluded that L-arginine and L-ornithine can act as precursors of the tropane alkaloids formed in young belladonna plants.

This experiment remains one of the enigmas of alkaloid biosynthesis studies. Its well-planned methodology, careful evaluation of results, and the agreement of the conclusions reached with those obtained using isotopes, do not permit it to be disregarded. Nevertheless, the fact remains that the root of the belladonna plant is the principal site of synthesis of the tropane alkaloids, and whether the increases in alkaloidal content observed in this experiment were actually due to leaf biosynthesis awaits experimental verification.

Andries, Fairbairn, and Youngken (15) have reported the results of experiments in which *Datura stramonium* plants grown in Hoagland and Arnon's solution were fed 0.2 g/l of tryptophan over a five-day growth period. The results, expressed on a dry weight basis, indicate a 25% increase in alkaloid but a 21% decrease in niacin over the controls. In a smiliar experiment conducted over a two-day period, a 33% increase in alkaloid was noted. Tryptophan feeding of excised *Datura* leaves had no appreciable effect on either niacin or alkaloid content. None of these results has been verified by any subsequent experiments with labeled tryphophan.

Distilled water was also substituted for the nutrient solution, and plants maintained in it for five days were compared to those placed in aqueous solutions of tryptophan for the same period. Under these conditions the tryptophan generally caused a decrease in alkaloid concentration in the plants, but this was dependent to some extent on whether or not the solutions were subjected to aeration.

The objections of Klein and Linser to root feeding of amino acids are now known to be largely invalid. Miettinen (16) has concluded that amino acids are taken up by plant roots without decomposition and that the assimilation may be regarded as a natural phenomenon. Furthermore, such amino acids are transported to all parts of the plant and are known to be used effectively for protein synthesis. Consequently, no objection can be raised to the method of root feeding of amino acids per se. However, under the conditions of the experiment of Andries *et al.* in which the roots of non-sterile plants were immersed in tryptophan-containing solution for an extended period of time, it must be questioned how much of the observed effects was due to the amino acid and how much to various decomposition products of it. The use of a non-sterile root feeding technique, coupled with the fact that analytical results are expressed only on a dry weight basis, make it difficult to give much credence to the increases in alkaloid content reported in this paper. Critical judgment of the work must await further studies aimed at evaluating the role of tryptophan or niacin in tropane synthesis. Preferably, these should be carried out with labeled precursors or with feedings of non-labeled tryptophan under sterile conditions.

In evaluating the role of non-isotopic feeding experiments in the study of alkaloid biosynthesis, it was originally decided to limit the survey to intact higher plants or at least to intact organ systems of

higher plants developed under conditions simulating a normal environment. This would exclude studies carried out with isolated enzyme systems, microorganisms, tissue homogenates, and with excised root or other tissue cultures. These biological systems do not present the investigator with the same type of problem, particularly with the complexities of absorption and transport of precursors and localization of active enzyme systems, as do intact plants or the major organs obtained from plants grown in their customary environment.

However, it becomes necessary to mention one experiment carried out with sterile root cultures since this furnishes biosynthetic evidence not otherwise available. Reinouts van Haga (17) has reported that when ornithine was fed to sterile root cultures of *Atropa belladonna*, the alkaloid content increased from 0.4% to 0.8% on a dry weight basis and that chromatographic analysis revealed an increase in both hyoscyamine and scopolamine. This would seem to confirm the role of ornithine as a precursor of both these tropane derivatives, a fact previously not established. Moreover, it indicates the ease with which clear-cut results (100% increase) can be obtained in experiments involving those tissues which possess the greatest synthetic ability.

Another feeding experiment involving an enzyme activator in addition to an added precursor has been carried out by Jindra *et al.* (18). *Datura stramonium* plants were grown in culture vessels containing sand and nutrient solution, and solutions of arginine were injected directly into their vascular systems. Injections were said to be made into the lower part of the shoot in such a manner that the solutions flowed into the plant of their own accord; however, details of the experimental procedure are not reported.

Plants were harvested after vegetative development ceased (sixteen weeks), the roots cut off, and the overground parts of a given group combined, dried, and analyzed. The authors state that no important differences in the growth of these plants were noted, although this statement was apparently based solely on visual evidence. Therefore, the percentage of alkaloids calculated on the basis of the dry weight of the plant material was assumed to give accurate information as to the changes in alkaloidal content.

Additions of increased concentrations of manganese and cobalt to the nutrient solution were also tested for their effect as arginase activators. Some of the increased alkaloidal yields obtained in these experiments are summarized in Table 1.

TABLE 1

| | Alkaloid Content % (dry weight) | | |
|---|---|---|---|
| | Overground Parts (excluding seeds) | Leaves | Seeds |
| Control Plants | 0.063 | 0.120 | 0.088 |
| Arginine-Injected Plants | 0.090 | 0.167 | 0.112 |
| Manganese Addition (five-fold) | 0.102 | 0.199 | 0.148 |

From these results it would appear that arginine injection increases the alkaloid concentration in *Datura stramonium;* however, it also points out the difficulty in distinguishing between increases of alkaloid content produced by direct (precursor feeding) and indirect (enzyme activation) means. Although no obvious differences in growth of the experimental and the control plants were noted, changes in the carbohydrate concentration of the respective tissues, which could account for considerable dry-weight variations, would not be noticeable upon inspection. These problems of proper sampling impart an equivocal significance to the reported results.

The relationship of nicotinic acid to trigonelline is obvious and has been confirmed in a recent study employing labeled compounds (19), but isotope studies have shown that in higher plants (20) tryptophan is not a precursor of nicotinic acid. This singular fact, which could not have been anticipated from the results of earlier studies carried out with molds, may be used as a criterion in judging the reliability of the results obtained in a feeding experiment carried out by Zeijlemaker (6).

After determining that the cut shoots of three-week-old pea seedlings (15 cm long, 7–8 leaves) were capable of synthesizing trigonelline, a series of 20 such cuttings, selected for uniformity, was placed in a multiple potometer of special design which was capable of measuring the quantity of liquid absorbed by them. The feeding solution consisted of a tenfold dilution of Hoagland and Shive's solution containing niacin (ca. 20 $\mu$g/ml) or other compounds (tryptophan, ornithine, citrulline, pyridoxine, pyruvic acid) to be tested for their influence on trigonelline synthesis. Feedings were carried out for a two-day period, after which the amount of precursor fed and the quantity of trigonelline formed were determined.

The results indicated that above a definite level the nicotinic acid absorbed by the leaves was quantitatively transformed into trigonelline. Increases in trigonelline content per plant were substantial, e.g., 484 $\mu$g per niacin-fed plant in comparison with 237 $\mu$g per control plant. The addition of tryptophan or the other potential precursors enumerated above, either separately or in combination, did not cause any increase in either nicotinic acid or trigonelline content. It was concluded that these compounds did not determine the rate of nicotinic acid synthesis.

This appears to be a model feeding experiment yielding results of a conclusive nature. The fact that tryptophan did not serve as a precursor to nicotinic acid, and the subsequent verification of this fact by isotope experimentation, permits one to ascribe considerable significance to the results obtained. The success of the experiment would appear to be due to the fact that analytical results were expressed on a suitable per-plant basis, and perhaps even more important, the author was dealing with a plant tissue which was extremely active with respect to its ability to synthesize trigonelline under the experimental conditions imposed.

For some time the function of lysine as a precursor of the $\alpha$-pyridone ring of ricinine stood in doubt, especially in view of the finding of Leete and Leitz (21) that nicotinic acid serves as a precursor of this alkaloid. Grimshaw and Marion (22) quote an unpublished work of Reist and Marion which led to the conclusion that lysine did not function in this

manner. Recently a publication by Tamir and Ginsburg (23) has shown conclusively that [2–$C^{14}$] labeled lysine fed hydroponically to seedlings of *Ricinus communis* is incorporated in the ricinine produced by the plant, as is $\alpha$-amino [$\epsilon$-$C^{14}$] adipic acid.

This finding places the non-isotopic feeding experiment of Bogdashevskaya (24) in a new perspective. This author infiltrated the leaves of intact castor plants with a 1% solution of lysine for six days and obtained a definite increase in ricinine in the plant, especially in the roots. Confirmation of the validity of this report by isotopic methods does much to establish the validity of experimental evidence obtained in properly designed non-isotope feeding experiments.

The results of isotope investigations have demonstrated that tyramine can serve as a precursor of N-methyltyramine and hordenine in germinating barley (25). Phenylalanine and tyrosine also proved capable of functioning as precursors of hordenine (26, 27), and methionine and formate were both shown to be capable of functioning in the methylation of tyramine (28, 29).

Non-isotopic feeding experiments were carried out by Rabitzsch (30) in a study of the biosynthesis and metabolism of hordenine in seedlings of *Hordeum vulgare*. After a germination period of five and one-half days, barley seedlings were fed with 0.5 *N* Knop's nutrient solution containing additions of tyrosine, tyramine, hordenine, candicine, anthranilic acid, and shikimic acid. These solutions were allowed to remain in contact with the roots of the seedlings for one day and then the plant material was separated and analyzed. Results were expressed as absolute content of compounds in the roots as well as on a percentage basis of these organs; content per 10 g of barley was also reported.

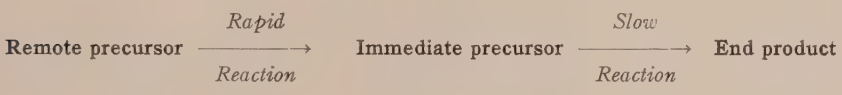
Feedings of tyrosine resulted in an extremely large increase of tyramine in the roots (700 $\mu$g increase per 10 g barley seed or 1200%). Both tyrosine and tyramine resulted in appreciable increases in hordenine content (76% and 224% respectively), but when hordenine was fed, it was demethylated as shown by a proportionate increase in tyramine and N-methyltyramine. Additions of candicine, anthranilic acid, and shikimic acid produced no noticeable effects on the amine content of the roots of the seedlings.

The results of the study confirm and expand the knowledge of hordenine metabolism in barley seedlings obtained by isotope studies. It also emphasizes the usefulness of the non-isotopic feeding method and the validity of results obtained with it, at least when it is applied to plants which are actively capable of synthesizing the compound being investigated.

To summarize the evidence obtained from a critical examination of all of these non-isotopic precursor feeding experiments, the primary difficulty appears to be the fact that in mature plants the percentage of tissue capable of active alkaloidal synthesis is very small. Subsequently, any increase in alkaloid production resulting from precursor feedings will be small in comparison to the weight or volume of the entire plant or even a single organ thereof. Too many uncontrollable variables affect the water balance, carbohydrate concentration, etc. of an entire plant to allow valid comparisons between the experimental plants and controls unless the increase in alkaloid is relatively large in comparison to the total weight of plant tissue employed.

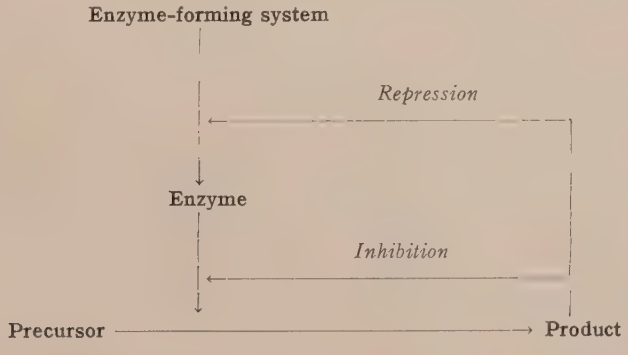
This would indicate that seedlings or very young plants which should theoretically contain a higher percentage of tissue capable of effecting alkaloid synthesis would be the most desirable experimental organisms for non-isotopic precursor feeding studies. In general, this has proven true experimentally; however, caution is necessary since in certain cases seedlings may lack the necessary synthetic abilities.

The difficulty in distinguishing between remote and immediate precursors in this type of feeding experiment must not be overlooked. A remote precursor may be converted rapidly to an immediate precursor which, even if fed as such, is converted very slowly by a rate-limiting reaction to the end product.




For the same reason it becomes difficult, if not impossible, to differentiate between precursors per se and other factors (enzyme activators) which may bring about an increase in the velocity of the rate-limiting reaction in the biosynthetic pathway.

The afore-mentioned rate-limiting mechanisms which render difficult the interpretation of results obtained in non-isotopic precursor feeding experiments are probably minor in comparison to the problems produced by the phenomenon of negative feedback. Although no experimental works have directly related this phenomenon to alkaloid biosynthesis, the Symposium on Modulation of Gene Expression by Chemical Feedback on Enzyme Synthesis and Activity, held at the 138th national meeting of the American Chemical Society in New York City in 1960, emphasized its extreme importance in a wide variety of biochemical reactions (31). In fact, chemical feedback in biological systems has been labeled by Dr. V. R. Potter as the most significant finding since the development of the gene concept.




If the amount of product of a chemical reaction in a biological system is increased by artificial means, the system may slow its reaction rate. This slowing can result from suppression of enzyme activity or of

enzyme formation. These two types of negative feedback are respectively designated as inhibition and repression. Feedback may also be of a positive nature, but negative feedback is believed to be the more important variety. Chemical feedback is thus an important phenomenon in which a product is capable of controlling its own rate of formation.

If negative feedback is operative in the systems which biosynthesize alkaloids in plants, the basic premise that administration of large quantities of precursor would lead to an appreciable increase in end product is in error. The scope and effectiveness of negative feedback control of reactions involved in alkaloid biosynthesis, including the influence of translocation of product from the site of synthesis, forms a suitable subject for future experimentation.

Although the fundamental objective of this review has been a critical survey of methodology, it has not been possible to separate this topic entirely from that of interpreting the results obtained by application of the methods. Differentiation of remote and immediate precursors, activation of enzyme systems, and the possible influence of negative feedback mechanisms have been given brief mention as factors which influence the evaluation of experimental data. Certainly other factors, such as the existence of natural precursor pools, the influence of cell permeability, the topographical separation within an organ of the biosynthetic apparatus, the connection between alkaloid production and growth activity, and a host of related subjects are equally worthy of consideration in any definitive treatment of data interpretation.

Certain non-isotopic precursor feeding experiments in the study of alkaloid biosynthesis have yielded results which are of unquestionable validity. The results of numerous other experiments of this type are equivocal, and some have been definitely proved to be invalid. The experimental and analytical requirements for a successful experiment of this sort are variable but extremely critical.

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